Appl Microbiol Biotechnol (1985) 22:41–45

Applied and Microbiology Biotechnology

Modification of fatty acid composition of lipid accumulating yeasts with cyclopropene fatty acid desaturase inhibitors

R. S. Moreton

Cadbury Schweppes Group Research, Lord Zuckerman Research Centre, University of Reading, Whiteknights, Reading, RG6 2LA, UK

Summary. The effect of cyclopropene fatty acids, sterculic and malvalic, on the lipids of yeasts grown under nitrogen limiting, lipid accumulating, conditions was studied. The ratio of stearic to oleic acid showed a dose response effect, with an increase in stearic acid content as the dose of cyclopropene fatty acid increased, and a corresponding reduction in oleic acid. Linoleic and linolenic acids were not affected to the same extent. These effects are shown for the yeasts *Candida* sp. 107, *Trichosporon cutaneum*, and *Rhodosporidium toruloides*.

Introduction

Oleaginous yeasts can accumulate large quantities of lipid when grown under nitrogen limiting conditions (Ratledge 1982). These lipids usually comprise >80% triglyceride, some partial glycerides and small quantities of polar lipids. Fatty acids of the glyceride fractions are principally 16 and 18 carbon acids showing Δ9, Δ12, and Δ15 desaturation, similar to those found in plant storage lipids, and further characterised by a low content of stearic acid (18:0), typically around 5% (w/w) and almost invariably below 10%, 40—50% oleic acid (18:1), 20—30% palmitic (16:0), the remainder usually being linoleic (18:2) and linolenic (18:3) acids in variable proportions. Some organisms show substantial quantities of palmitoleic acid (16:1). The fatty acid composition of such yeasts is remarkably consistent and reproducible. Changes in growth conditions and medium constituents have only minor effects on fatty acid composition.

Offprint requests to: R. S. Moreton

The cyclopropene fatty acids sterculic and malvalic, found in seed oils of the *Malvaceae* and *Sterculiaceae* families (Christie 1970; Yano 1972), are 17 and 18 carbon straight chain fatty acids respectively with a cyclopropene group in the Δ9 position. They have been shown to interfere with fatty acid desaturation in animals (Shenstone et al. 1965, Johnson et al. 1967; Raju and Reiser 1967), plants (James et al. 1968), and certain yeasts (Reiser et al. 1963).

Neither plants, animals nor microorganisms produce unsaturated fatty acids directly. They are produced from a saturated 'parent' by sequential desaturation, double bonds being introduced first at the Δ9 position, then at Δ12 and Δ15. These three desaturations are catalyzed by three separate desaturases specific for the Δ9, Δ12 and Δ15 positions. Desaturation proceeds by an oxidative mechanism requiring molecular oxygen, NADH, NADPH and substrate. It is known in plants (McKeon and Stumpf 1982) that the substrate is either the acyl carrier protein or coenzyme A thiolester of the fatty acid, and it has been recently shown in yeasts that membrane bound phospholipids can also be desaturase substrates (Ferrante et al. 1983).

We were interested in determining if the cyclopropene fatty acids could be used to modify yeast lipids to produce industrially important lipids, such as those described in U.S. Patent 4,308,350 and U.K. Patent Application 2091286 A, produced by growing yeasts on fatty acid derivatives.

Methods

Materials. Kapok seed oil containing 0.7% sterculic and 2.6% malvalic acids was obtained from the Tropical Products Insti-

tute, London. Sterculia oil was obtained from Hindustan Cocoa Products, India, and contained 49.8% sterculic and 4.8% malvalic acids. Purified methyl sterculate was prepared from sterculia oil, and contained 91.9% methyl sterculate, the remainder being oleic acid.

Lipid extraction. Yeast cultures were harvested by centrifugation (10,000 g for 15 min) to recover cells, washed with distilled water, resuspended and centrifuged again under the same conditions and freeze dried. Approximately 0.5 g of freeze dried cells were suspended in 100 ml of $CHCl_3$/MeOH (2 : 1 v/v) for 24 h, filtered through Whatman No. 1 filter paper and added to 100 ml of 5% (w/v) Na_2SO_4 solution in a separating funnel, shaken vigorously and left overnight for the phases to separate. The $CHCl_3$ phase was removed, the solvent evaporated and the lipid recovered and weighed.

Lipid analysis. Approximately 50 mg of lipid was saponified with 1.5 ml of 5% (w/v) NaOH in methanol at 100° C, 1.5 ml of BF_3/methanol (Sigma) added and heated at 80° C for 15 min. To this was added 1 ml saturated NaCl solution and 1 ml hexane, and the mixture was shaken vigorously, centrifuged, and the hexane layer removed.

Fatty acid composition was determined by gas liquid chromatography of the methyl esters on a 2 m × 4 mm i.d. glass column of 10% DEGS on Chromosorb W. HP (Phase Separations) at 175° C using nitrogen as the carrier gas, on a Pye-Unicam PU 4500 chromatograph with a Hewlett-Packard 3390 A integrator. Cyclopropene fatty acids were determined by GLC analysis of their methyl esters treated with $AgNO_3$-saturated methanol, as described by Schneider et al. (1968). Peak areas of the ether and ketone derivatives formed by this procedure were added to give total CPFA (cyclopropene fatty acid) content.

Growth conditions. Organisms were cultivated for 4 days in 250 ml baffled shake flasks containing 50 ml medium, using an orbital incubator at 150 r.p.m. and 30° C. The growth medium contained the following constituents ($g\,l^{-1}$), glucose, 30, NH_4Cl, 0.5, KH_2PO_4, 7.0, Na_2HPO_4, 2.0, $MgSO_4 \cdot 7H_2O$, 1.5, $CaCl_2 \cdot 2H_2O$, 0.1, $FeCl_3 \cdot 6H_2O$, 0.008, $ZnSO_4 \cdot 7H_2O$, 0.0001, yeast extract, 1.5, initial pH, 5.5. This medium is nitrogen limited with a C : N ratio of 34.53, assuming the yeast extract to be 14.5% N. The cyclopropene containing oils, or the purified methyl sterculate, were added to the shake flasks prior to sterilisation by autoclaving.

Organisms. Cultures used in this study were *Candida* sp. 107, NCYC 911, *Trichosporon cutaneum,* NCYC 1422, *Lipomyces lipofer,* NCYC 982 and *Lipomyces starkeyi,* NCYC 692, from the National Collection of Yeast Cultures, Norwich, UK, *Rhodosporidium toruloides,* IFO 0559, from the Institute for Fermentation, Osaka, Japan, and *Lipomyces starkeyi,* CBS 6142, CBS 6047, CBS 0678, CBS 6132 and CBS 1807 from the Centraalbureau voor Schimmelcultures, Delft, Holland, and baker's yeast, *Saccharomyces cerevisiae* types DCL (Distillers, UK) and Fermipan (Gist-Brocades, Holland) both obtained commercially.

Results

Lipids from cultures of *Candida* sp. 107, *Trichosporon cutaneum,* and *Rhodosporidium toruloides,* were extracted and analysed for fatty acid composition after growth with and without the addition of known quantities of oils containing cyclopropene fatty acids, and a partially purified preparation of the methyl ester of one of these, methyl sterculate.

Fatty acid composition of the lipid from *Candida* sp. 107 grown with kapok oil, sterculia oil and methyl sterculate, and the change in the ratio of 18 : 0/18 : 1 caused by these additives, and the degree of unsaturation of the lipid, are shown in Tables 1—3.

All three compounds caused substantial changes in the fatty acid composition of this organism. Palmitoleic and oleic acids were reduced and stearic increased, with increasing doses of CPFA. Quantitatively, the decrease in oleic was virtually the same as the increase in stearic. For example, at a dose of 1.6 $ml \cdot l^{-1}$ kapok oil, stearic increased by 29.0% and oleic decreased by 28.0%, compared with the control values. Similar quantitative effects were seen at all doses of the three compounds. Since the concentration of palmi-

Table 1. Effect of kapok oil on fatty acid composition of *Candida* sp. 107

Kapok oil ($ml \cdot l^{-1}$)	Dose CPFA ($ml \cdot l^{-1}$)	Biomass ($g \cdot l^{-1}$)	Lipid ($g \cdot l^{-1}$)	Lipid (% w/w)	Fatty acid composition (% w/w)						Ratio (18:0/18:1)	ΔU
					16:0	16:1	18:0	18:1	18:2	18:3		
0	0	9.75	4.17	42.87	19.5	3.5	3.9	38.0	30.91	<1.0	0.1	3.1
0.1	0.0033	9.10	4.02	44.19	23.4	1.1	11.3	26.0	32.38	<1.0	0.4	1.7
0.2	0.0066	9.05	3.40	37.58	25.3	<1.0	15.9	21.0	30.49	<1.0	0.8	1.3
0.3	0.0099	6.85	2.86	41.92	27.6	<1.0	20.0	18.0	28.13	<1.0	1.2	1.0
0.4	0.0132	9.78	3.84	39.34	26.1	<1.0	22.2	15.3	29.42	<1.0	1.5	0.9
0.8	0.0264	10.63	4.29	40.40	24.3	<1.0	20.9	16.3	31.63	<1.0	1.3	1.1
1.2	0.0396	8.32	3.55	42.67	25.7	<1.0	29.9	11.3	25.99	<1.0	2.7	0.7
1.6	0.0528	9.71	3.37	39.82	26.8	<1.0	32.9	10.0	25.09	<1.0	3.3	0.6
2.0	0.1056	7.38	3.04	41.19	25.7	<1.0	31.0	10.1	26.18	<1.0	3.1	0.6

Degree of unsaturation $\Delta U = \frac{(18:1 + 18:2 + 18:3)}{(18:0 + 16:0)}$

Table 2. Effect of sterculia oil on fatty acid composition of *Candida* sp. 107

Sterculia oil ($ml \cdot l^{-1}$)	Dose CPFA ($ml \cdot l^{-1}$)	Biomass ($g \cdot l^{-1}$)	Lipid ($g \cdot l^{-1}$)	Lipid (% w/w)	Fatty acid composition (% w/w)						Ratio (18:0/18:1)	ΔU
					16:0	16:1	18:0	18:1	18:2	18:3		
0	0	9.90	3.05	30.85	27.7	2.5	5.2	32.9	27.2	<1.0	0.2	1.8
0.04	0.0216	4.71	0.86	17.67	28.3	2.4	24.4	11.9	30.4	<1.0	2.1	0.8
0.10	0.0540	5.25	1.19	21.29	29.3	<1.0	28.5	9.8	25.0	<1.0	2.9	0.6
0.20	0.1080	6.04	1.51	24.70	28.2	<1.0	32.9	10.3	23.0	<1.0	3.2	0.5
0.40	0.2160	6.70	1.82	26.76	28.7	<1.0	36.7	10.1	18.9	<1.0	3.6	0.4
0.80	0.4320	5.15	1.17	21.99	29.8	<1.0	28.0	11.1	22.7	<1.0	2.5	0.6
1.20	0.6480	4.78	1.00	20.78	25.4	<1.0	35.1	8.8	24.2	<1.0	4.0	0.5
1.60	0.8640	5.97	1.58	26.44	25.3	<1.0	44.6	7.9	13.8	<1.0	5.6	0.3
2.00	1.0800	7.08	2.65	33.09	24.8	<1.0	43.7	9.9	14.3	<1.0	4.4	0.4
4.00	2.1600	8.23	3.26	39.38	26.8	<1.0	40.4	8.3	10.1	<1.0	4.9	0.3

Degree of unsaturation $\Delta U = \frac{(18:1+18:2+18:3)}{(18:0+16:0)}$

Table 3. Effect of methyl sterculate on fatty acid composition of *Candida* sp. 107

Methyl sterculate ($ml \cdot l^{-1}$)	Dose CPFA ($ml \cdot l^{-1}$)	Biomass ($g \cdot l^{-1}$)	Lipid ($g \cdot l^{-1}$)	Lipid (% w/w)	Fatty acid composition (% w/w)						Ratio (18:0/18:1)	ΔU
					16:0	16:1	18:0	18:1	18:2	18:3		
0	0	11.66	4.34	37.10	26.2	3.0	5.5	35.3	20.9	<1.0	0.2	1.8
0.02	0.0184	11.69	4.28	36.50	30.5	3.1	6.6	34.4	18.3	<1.0	0.2	1.3
0.05	0.0459	12.64	4.59	36.28	32.2	1.0	10.2	23.3	11.4	<1.0	0.4	0.8
0.10	0.0919	11.16	4.16	37.20	31.0	1.7	9.7	30.1	20.7	<1.0	0.3	1.2
0.20	0.1838	11.06	3.99	36.05	32.6	0.9	15.6	23.3	19.4	<1.0	0.7	0.9
0.50	0.4595	10.32	3.49	33.88	30.1	0.3	26.4	16.6	18.9	<1.0	1.6	0.6
1.00	0.9190	10.49	3.78	36.14	30.6	0.1	31.6	13.5	15.2	<1.0	2.3	0.5
2.00	1.838	8.10	2.51	29.75	30.4	0.2	39.1	4.1	3.0	<1.0	9.5	0.1

Degree of unsaturation $\Delta U = \frac{(18:1+18:2+18:3)}{(18:0+16:0)}$

toleic is low in this organism (control values ~3.0%) reductions in palmitoleic, were not significant compared to the reduction in oleic, otherwise it would be necessary to compare the total reduction in palmitoleic and oleic with the reduction in stearic. Concentrations of palmitic remained similar to the control values throughout, whereas linoleic (18:2), showed a reduction with increasing doses of kapok oil which was much more pronounced with sterculia oil which at the

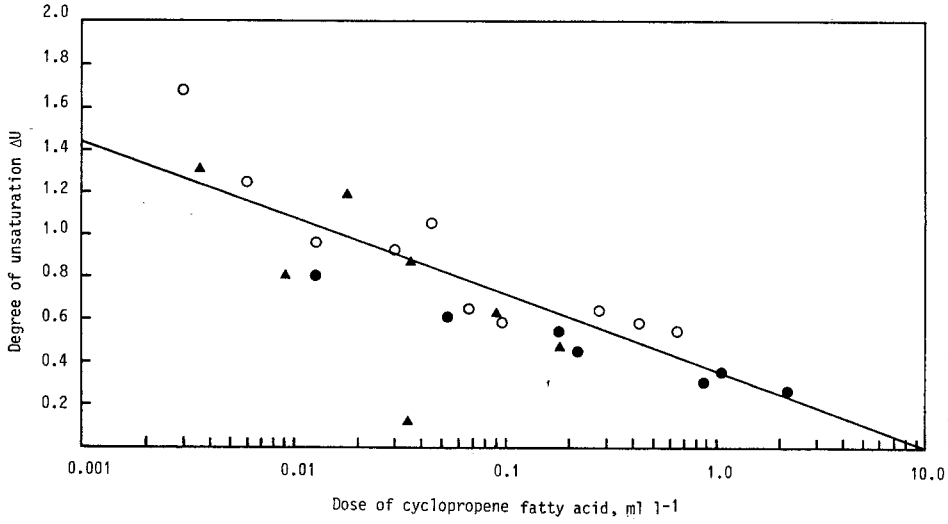

Fig. 1. Relationship between dose of cyclopropene fatty acid and degree of lipid unsaturation, ΔU;

$\Delta U = \frac{(18:1+18:2+18:3)}{(18:0+16:0)}$

(▲) methyl sterculate, (○) kapok oil, (●) sterculia oil

highest dose used reduced 18 : 2 from > 27.0% to 10.1%. Concentrations of linolenic (18 : 3) were below 1% with this organism, including controls. The results expressed as changes in the ratio of 18 : 0/18 : 1 or as the degree of unsaturation of the lipid are shown in the tables.

A plot of CPFA concentration, calculated from the % CPFA in each of the sources, versus the degree of unsaturation of the lipid (18 : 1 + 18 : 2 + 18 : 3/18 : 0 + 16.0), ΔU, gives a straight line relationship on a logarithmic scale, showing that the effect is due to the CPFA and not another constituent of the oils (Fig. 1).

Biomass and lipid yields were generally similar to the controls, but with some evidence of a reduction in biomass at the highest addition levels of CPFA. Lipid content of the cells (% w/w) was fairly constant except with sterculia oil, where some variation was seen.

Similar effects were observed with both kapok and sterculia oils with *Trichosporon cutaneum* and *Rhodosporidium toruloides*. Tables 4 and 5 show the effect of sterculia oil on the lipid composition of these organisms.

Addition of CPFAs inhibit the Δ9 desaturase and reduces both oleic and palmitoleic acid concentrations in these oleaginous yeasts. The expected consequential reduction of 18 : 2 and 18 : 3 was not observed with all of the organisms examined. With *Candida* sp. 107, and *Rhodosporidium toruloides*, 18 : 2 was substantially reduced by the addition of sterculia oil, whereas the 18 : 3 showed no reduction. *Candida* sp. 107, has a normal value of < 1.0% 18 : 3 and *Rhodosporidium toruloides*, 5—6%, 18 : 3 respectively. With *Trichosporon cutaneum*, 18 : 2 showed no reduction on addition of sterculia oil, and even a small increase in some cases with kapok oil. The concentration of 18 : 3 remained constant at 1—2%.

Not all organisms responded in the same way as those described above. Several strains of *Lipomyces* would not grow in the presence of CPFA,

Table 4. Effect of sterculia oil on fatty acid composition of *Trichosporon cutaneum*

Sterculia oil ($ml \cdot l^{-1}$)	Dose CPFA ($ml \cdot l^{-1}$)	Biomass ($g \cdot l^{-1}$)	Lipid ($g \cdot l^{-1}$)	Lipid (% w/w)	Fatty acid composition (% w/w)						Ratio (18:0/18:1)	ΔU
					16:0	16:1	18:0	18:1	18:2	18:3		
0	0	9.88	2.78	29.38	28.5	< 1.0	6.5	49.6	13.3	1.2	0.1	1.8
0.04	0.0216	8.33	2.58	31.08	30.5	< 1.0	19.0	32.7	16.0	1.00	0.6	1.0
0.10	0.0540	8.10	2.56	31.55	30.6	< 1.0	20.8	29.5	16.2	1.2	0.7	0.9
0.20	0.1080	8.40	2.54	30.21	32.1	< 1.0	23.8	26.5	14.1	1.0	0.9	0.8
0.40	0.2160	8.26	2.58	31.15	32.1	< 1.0	23.1	25.9	14.5	1.5	0.9	0.8
0.80	0.4320	7.60	2.29	30.17	33.5	< 1.0	22.1	24.9	15.1	1.8	0.9	0.8
1.20	0.6480	8.11	2.67	30.53	35.3	< 1.0	22.9	24.1	13.5	1.7	0.9	0.7
1.60	0.8640	8.20	2.54	30.99	34.2	< 1.0	23.4	25.2	12.9	1.4	0.9	0.7
2.00	1.0800	7.63	2.35	30.79	35.0	< 1.0	22.9	25.4	12.9	1.6	0.9	0.7
4.00	2.1600	8.32	2.61	31.48	35.1	< 1.0	22.7	23.0	14.6	1.6	1.0	0.7

Degree of unsaturation $\Delta U = \frac{(18:1 + 18:2 + 18:3)}{(18:0 + 16:0)}$

Table 5. Effect of sterculia oil on fatty acid composition of *Rhodosporidium toruloides*

Sterculia oil ($ml \cdot l^{-1}$)	Dose CPFA ($ml \cdot l^{-1}$)	Biomass ($g \cdot l^{-1}$)	Lipid ($g \cdot l^{-1}$)	Lipid (% w/w)	Fatty acid composition (% w/w)						Ratio (18:0/18:1)	ΔU
					16:0	16:1	18:0	18:1	18:2	18:3		
0	0	12.33	3.70	30.05	16.2	< 1.0	3.5	42.0	28.5	5.0	0.1	3.8
0.04	0.0216	8.48	2.65	29.97	15.1	< 1.0	17.1	19.0	17.0	8.8	0.9	1.4
0.10	0.0540	10.14	3.38	32.57	21.5	< 1.0	22.0	24.1	17.9	8.0	0.9	1.2
0.20	0.1080	10.13	3.56	33.82	15.1	< 1.0	31.5	22.6	14.6	6.7	1.4	0.9
0.40	0.2160	8.81	2.58	28.95	17.3	< 1.0	31.7	17.1	17.0	9.2	1.9	0.9
0.80	0.4320	10.80	3.82	35.39	17.6	< 1.0	31.0	24.1	15.8	6.2	1.3	1.0
1.20	0.6480	10.45	2.27	22.53	17.0	< 1.0	30.7	24.1	15.9	4.8	1.3	0.9
1.60	0.8640	11.08	2.86	25.95	15.1	< 1.0	37.8	19.5	16.2	6.4	1.9	0.8
2.00	1.0800	10.29	3.29	31.94	12.9	< 1.0	40.9	18.0	14.9	6.1	2.3	0.7
4.00	2.1600	11.63	3.57	30.31	14.5	< 1.0	35.3	19.6	16.1	6.2	1.8	0.8

Degree of unsaturation $\Delta U = \frac{(18:1 + 18:2 + 18:3)}{(18:0 + 16:0)}$

and among those that would, there was no systematic alteration in the fatty acid composition. Neither of the two strains of *Saccharomyces cerevisiae* tested showed an increase in 18:0 with CPFAs.

Discussion

The initial observation of the effect of CPFA by Reiser and co-workers (1963) used a culture of *S. cerevisiae* described as American type culture LK2GL2, grown on YEPG medium under carbon limiting conditions with 0.1 $ml \cdot l^{-1}$ sterculia oil. They showed a considerable reduction in palmitoleic and oleic levels with the addition of sterculia oil, but, curiously, little effect on stearic with this organism. *S. cerevisiae* does not accumulate lipid to any appreciable extent, even when grown under nitrogen limitation and is unusual in that most strains have 15—16% 16:1. Neither 18:0, 18:1 nor 16:1 were affected by CPFAs with our strains of *S. cerevisiae* either.

Assuming the CPFA is transported into the cell, these results immediately pose the question as to why such differences should occur and why these compounds are lethal to some members of the species *Lipomyces* and not others. Little information is available on the detailed biochemistry of lipid desaturation in yeasts, but these results suggest the possibility of more than one desaturase system, even in members of the same species, with a differing response to this type of inhibitor. One system may be present in some species and the other or both systems in other species.

We have not attempted to fractionate storage lipid and structural lipid to examine the composition of the latter relative to the overall lipid profile reported here. If the structural (membrane) lipid is affected in the same way as storage lipid by CPFAs, it would seem reasonable to suppose that biomass would have been reduced in response to a reduction in membrane fluidity caused by the incorporation of more saturated fatty acids into membranes. This was not observed at the highest addition levels of CPFAs, although a reduction in biomass was observed on addition of low levels of CPFAs in some cases, suggesting some form of adaptation.

Acknowledgement. The author thanks M. H. Jee of this laboratory for preparing the purified methyl sterculate.

References

Christie WW (1970) Cyclopropane and cyclopropene fatty acids. In: Topics in lipid chemistry, vol I. Edited by FD Gunstone. Logos Press, London

Ferrante G, Ohno Y, Kates M (1983) Influence of temperature and growth phase on desaturase activity of the mesophilic yeast *Candida lipolytica*. Can J Biochem Cell Biol 61:171—177

James AT, Harris P, Bezard J (1968) The inhibition of unsaturated fatty acid biosynthesis in plants by sterculic acid. Eur J Biochem 3:318—325

Johnson AR, Pearson JA, Shenstone FS, Fogerty AC (1967) Inhibition of the desaturation of stearic to oleic acid by cyclopropene fatty acids. Nature 214:1244—1245

McKeon TA, Stumpf PK (1982) Purification and characterisation of the stearoyl-acyl carrier protein desaturase and the Acyl-acyl carrier protein thioesterase from maturing seeds of safflower. J Biol Chem 257:12141—12147

Ratledge C (1982) Microbial oils and fats. An assessment of their commercial potential. Prog Ind Microbiol 16:119—206

Raju PK, Reiser R (1967) Inhibition of fatty acyl desaturase by cyclopropene fatty acids. J Biol Chem 242:379—384

Reiser R, Parekh CK, Meinke WW (1963) The metabolism of cyclopropene fatty acids. In: Frazer AC (ed) Biological problems of lipids, vol I. Elsevier, New York

Schneider EL, Loke SP, Hopkins DT (1968) Gas-liquid chromatographic analysis of cyclopropenoid fatty acids. J Am Oil Chem Soc 45:585—590

Shenstone FS, Vickery JR, Johnson AR (1965) Studies on the chemistry and biological effects of cyclopropenoid compounds. J Agric Food Chem 13:410—414

Yano I, Nicholls BW, Morris LJ, James AT (1972) The distribution of cyclopropane and cyclopropene fatty acids in higher plants *(Malvaceae)*. Lipids 7:30—34

Received September 11, 1984